

HIV protease inhibitor nelfinavir inhibits replication of SARS-associated coronavirus

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Abstract

A novel coronavirus has been identified as an etiological agent of severe acute respiratory syndrome (SARS). To rapidly identify anti-SARS drugs available for clinical use, we screened a set of compounds that included antiviral drugs already in wide use. Here we report that the HIV-1 protease inhibitor, nelfinavir, strongly inhibited replication of the SARS coronavirus (SARS-CoV). Nelfinavir inhibited the cytopathic effect induced by SARS-CoV infection. Expression of viral antigens was much lower in infected cells treated with nelfinavir than in untreated infected cells. Quantitative RT-PCR analysis showed that nelfinavir could decrease the production of virions from Vero cells. Experiments with various timings of drug addition revealed that nelfinavir exerted its effect not at the entry step, but at the post-entry step of SARS-CoV infection. Our results suggest that nelfinavir should be examined clinically for the treatment of SARS and has potential as a good lead compound for designing anti-SARS drugs.

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Severe acute respiratory syndrome (SARS) is an emerging disease that was first reported in Guangdong Province, People's Republic of China, in November, 2002. Since then, SARS has spread to 32 countries and has resulted in more than 800 deaths from respiratory distress syndrome [1–3]. An overall estimate of case fatality reached 14–15% as reported by WHO [4] and the mortality rate in people older than 60 years could be as high as 43–55% [5].

Several groups, including the authors, isolated a novel coronavirus from SARS patients [2,6,7]. It has been shown that SARS-CoV satisfies Koch's postulates for causation—its consistent isolation from patients suffering from SARS, isolation of the virus and reproduction of disease in non-human primates after inoculation, and the presence of a specific antibody response against the virus in both SARS patients and artificially infected primates [8]. Now its etiological role in SARS is widely accepted.

The outbreak of SARS in several countries has led to the search for active antiviral compounds and vaccines for this disease [9]. Although the results of many clinical

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experiments have been reported, no consensus on treatment has been reached to date. Therapeutic protocols with steroids and ribavirin have been widely used empirically from the outset of the epidemic [10,11]. The use of steroids for SARS seemed beneficial, whenever they are appropriately applied. However, the optimal timing, dosage, and duration of treatment have not yet been determined. On the other hand, the administration of ribavirin did not apparently reduce either the rate of intratracheal intubation or that of mortality [12]. Moreover, significant toxicity, such as hemolytic anemia, has been attributed to ribavirin [13]. A few preliminary trials and in vitro data suggested the possibility of treating SARS with interferon [14–16]. Other agents including glycyrrhizin and convalescent plasma require further studies [17]. As is well established in the case of HIV-1 infection, the combination of antiviral drugs will make it possible to establish a better protocol for the treatment of SARS.

To identify anti-SARS drugs available for clinical use as rapidly as possible, we screened a set of compounds including antiviral drugs already in human clinical use. We found that nelfinavir, a widely used HIV-1 protease inhibitor, could inhibit SARS-CoV replication efficiently. Our results suggest that nelfinavir should be examined clinically for the treatment of SARS.

Materials and methods

Cell culture and virus. Vero E6 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and glutamine–streptomycin solution in 5% CO₂ in humidified air at 37 °C.

The FFM-1 strain of SARS-CoV was isolated from a SARS patient admitted to the Clinical Centre of Frankfurt University. This strain was used in all experiments to assess the antiviral activity of the drugs.

Compounds for screening. A set of compounds for screening consisted of 24 drugs as follows: nelfinavir, saquinavir, KNI-272, TYA5, TYB5, ritonavir, lopinavir, indinavir, 4F-benzoyl-TN14003, 4F-benzoyl-TE14011, TN14003, T140, TC14012, FC131, T22, SDF-1, vMIP-II, TAK-779, SC34, N36, T-20, glycyrrhizin, glycyrrhetic acid, and Cardran sulfate.

Cytopathic effect assay. SARS-CoV was inoculated into a monolayer of Vero E6 cells in 24-well plates at a multiplicity of infection (MOI) of 0.01. The plates were incubated at 37 °C in 5% CO₂ for 3 days and CPE in each well was observed.

Immunofluorescence assay. The Vero E6 cells in 24-well plates were infected with SARS-CoV at the MOI of 0.01. The infected cells were fixed with methanol 24 h after infection and incubated at room temperature for 1 h with diluted serum sample from a SARS patient. After washing with PBS, the cells were incubated with anti-human-IgG antibody conjugated with FITC for 30 min at room temperature. The cells were washed with PBS, mounted in buffered glycerol, cover-slipped, and viewed with a fluorescence microscope.

RNA extraction and real-time RT-PCR assay. SARS-CoV RNA in the culture supernatant was purified with ISOGEN (Nippongene) according to the manufacturer's protocol. For quantification of SARS-CoV ORF-1 RNA, we performed real-time RT-PCR with the primers and the probe as follows: ORF1-F, AGCTACGAGCACCAGACACC; ORF1-R, ACTTTGGGCATTCCTTT; ORF1-probe, TCGAAA TTAAGAGTGCCAAGAAATTTGACACTTT. The fluorescence intensity generated from the probe was detected by the ABI-7700 sequence detector system (Applied Biosystems).

MTT assay. Vero E6 cells in 96-well plates were infected with SARS-CoV at the MOI of 0.01. After 36 h of culture, cells were incubated for 4 h in the presence of 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Formazan crystals were dissolved with 100 µL of 0.04 N HCl-isopropyl alcohol (acid isopropanol) and absorbance at 570 nm was measured with a reference wavelength of 655 nm.

Time-of-addition experiments. Drugs, including nelfinavir, were added to cultures of Vero E6 cells at the time of infection or 3 h after infection. Samples were processed for a quantitative RT-PCR assay and an immunofluorescence assay 24 h after infection. The cytopathic effect of infected cells was analyzed 36 h after infection.

Entry inhibition assay. Vero E6 cells were pretreated with each drug for 3 h, and SARS-CoV was inoculated at the MOI of 0.1. Cells and viruses were incubated for 3 h and washed with PBS three times. Subsequently, infected cells were lysed with ISOGEN (Nippongene) and RNA was purified according to the manufacturer's protocol. Extracted RNA samples were subjected to real-time RT-PCR analysis for quantification of SARS-CoV RNA as described above. As a loading control for normalization, 18S ribosomal RNA was quantified with the primers and the probe as follows: 18S-F, GTAACCCGTTGAACCCCAT; 18S-R, CCATCCAATCGGTAGTAGCG; and 18S-probe, TGCGTT GATTAAGTCCCTGCCCTTTGTA.

Results and discussion

Nelfinavir inhibited replication of SARS-CoV

We screened our chemical library and found that nelfinavir could inhibit SARS-CoV replication in Vero E6 cells. Nelfinavir clearly inhibited the cytopathic effect (CPE) induced by infection with SARS-CoV (Fig. 1A). We also examined the replication of SARS-CoV by immunofluorescence assay (IFA) with a serum sample from a patient with SARS. Expression of viral antigens was much lower in infected cells treated with nelfinavir than in untreated infected cells (Fig. 1B). Furthermore, we assessed the effect of nelfinavir on the production of virions. Nelfinavir significantly blocked the production of virions as revealed by quantitative RT-PCR (Fig. 2). By the use of MTT assay, we determined the concentration of the compound that reduced cell viability to 50% (CC₅₀), the concentration of the compound required for inhibition of CPE to 50% of the control value (EC₅₀), and the selectivity index (SI). Nelfinavir inhibited SARS-CoV replication at non-toxic doses with an approximate SI of 300, while the other inhibitors against HIV-1 protease (ritonavir, lopinavir, saquinavir, indinavir, TYA5, TYB5, and KNI-272) did not affect the replication of SARS-CoV (Table 1). These results revealed that nelfinavir is active in inhibiting SARS-CoV replication.

Nelfinavir inhibited SARS-CoV replication at the post-entry, but not the entry step

To disclose the step at which nelfinavir affects the virus life cycle, we performed time-of-addition experiments on the replication of SARS-CoV.

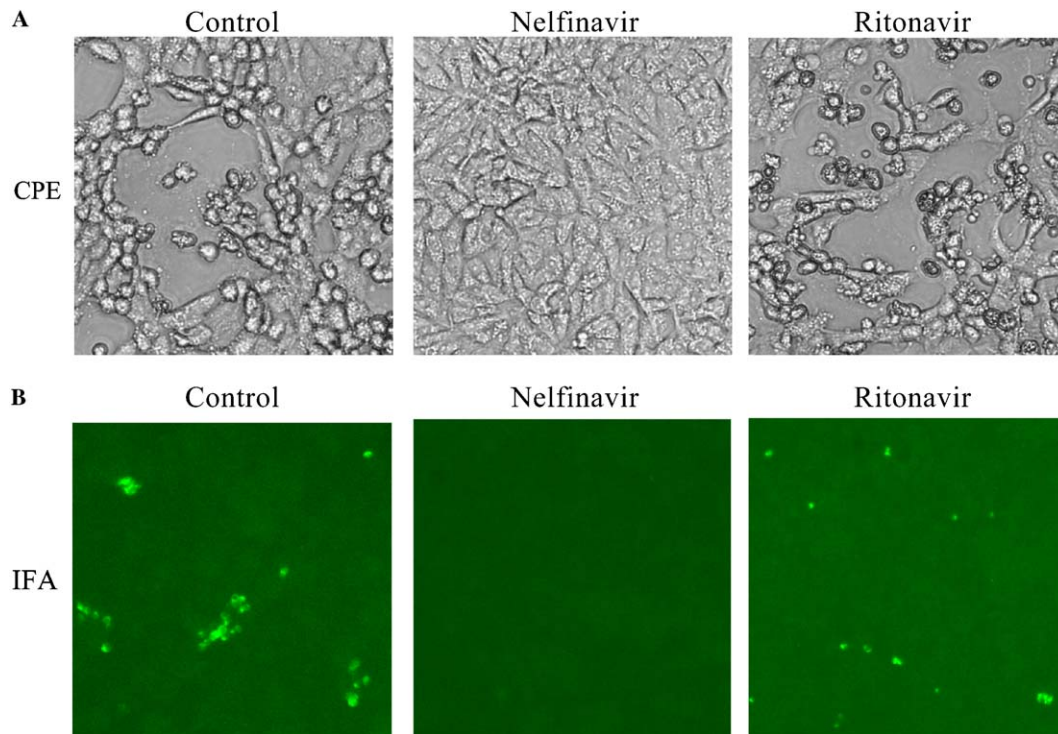


Fig. 1. Effect of nelfinavir on replication of SARS-CoV in Vero E6 cells. (A) Cytopathic effect (CPE) in Vero E6 cells. The cells were treated with phosphate-buffered saline (PBS) as a control, 10 μ M nelfinavir, or 10 μ M ritonavir for 3 h before infection. CPE was observed 36 h after infection. (B) Immunofluorescence assay (IFA) of infected cells treated with PBS, 10 μ M nelfinavir, or 10 μ M of ritonavir. Cells were fixed with methanol 24 h after infection and stained with serum samples from SARS patients.

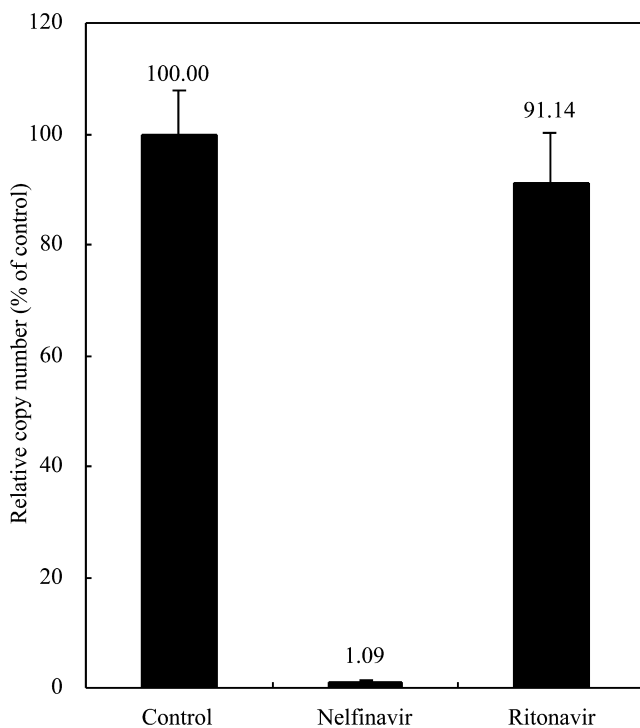


Fig. 2. Real-time RT-PCR for SARS-CoV RNA. Vero E6 cells had been treated with nelfinavir or ritonavir for 3 h before infection at the concentration of 10 μ M. Instead of these drugs, PBS was added as a negative control. Viral RNA in the culture supernatant was collected 24 h after infection and quantified by the use of a fluorogenic probe. All samples were analyzed in triplicate.

Table 1

Activity of compounds against SARS-associated coronavirus in Vero cell cultures

Compound	EC ₅₀ (μ M)	CC ₅₀ (μ M)	Selectivity index
Nelfinavir	0.048 (0.024)	14.5 (2.75)	302.1
Saquinavir	NC	31.4 (7.82)	NC
KNI-272	NC	8.85 (2.05)	NC
TYA5	NC	16.3 (3.13)	NC
TYB5	NC	9.22 (2.25)	NC
Ritonavir	NC	13.8 (2.94)	NC
Lopinavir	NC	24.15 (5.01)	NC
Indinavir	NC	9.63 (3.11)	NC

NC, not calculable.

EC₅₀, effective concentration of compound needed to inhibit the cytopathic effect to 50% of control value.

CC₅₀, cytotoxic concentration of the compound that reduced cell viability to 50%.

Mean (standard error) of three assays was calculated for each drug.

Nelfinavir significantly inhibited SARS-CoV replication when used before infection (Figs. 1A and B and 2). When this drug was added at the time of infection or 3 h after infection, it was still able to block the CPE induced by SARS-CoV infection (Fig. 3A). Addition of nelfinavir at various timings inhibited the expression of viral antigens in Vero cells as shown by IFA (Fig. 3B). Nelfinavir blocked the production of virions when used to treat the cells at the time of infection or 3 h after infection (Fig. 4). The other protease inhibitors including ritonavir had no effect on replication of SARS-CoV

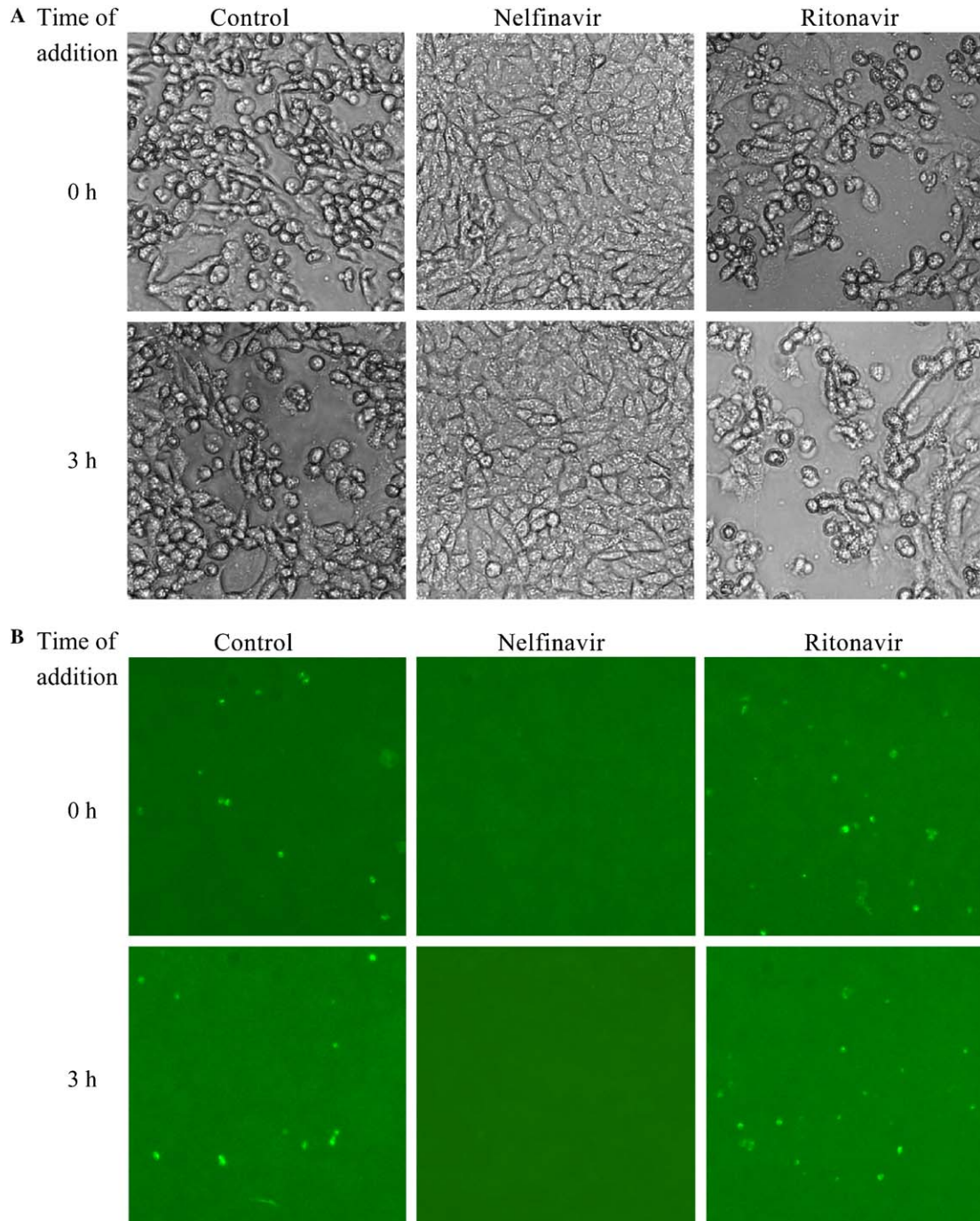


Fig. 3. Effect of various timings of drug addition on SARS-CoV replication in Vero E6 cells. (A) CPE in Vero E6 cells when drugs were added at the time of infection or 3 h after infection. The cells were treated with phosphate-buffered saline (PBS) as a control, 10 μ M nelfinavir, or 10 μ M ritonavir and CPE was observed 36 h after infection. (B) IFA of infected cells when drugs were added at the time of infection or 3 h after infection. The cells were treated with PBS as a control, 10 μ M nelfinavir, or 10 μ M ritonavir. The cells were fixed with methanol 24 h after infection and stained with serum samples from SARS patients.

(Figs. 3A and B and 4). These results indicate that the target(s) of nelfinavir may be involved in the post-entry step of SARS-CoV replication.

To investigate whether or not nelfinavir can affect the efficiency of virion entry, we quantified the copy number of SARS-CoV RNA in Vero cells immediately after the entry of virions.

Real-time RT-PCR revealed that nelfinavir did not affect the entry step of SARS-CoV infection (Fig. 5), which is consistent with our assumption that nelfinavir blocks the post-entry step of SARS-CoV replication.

The mechanisms that underlie the inhibitory action of nelfinavir on SARS-CoV replication remain to be identified. The main proteinase of SARS-CoV is one of

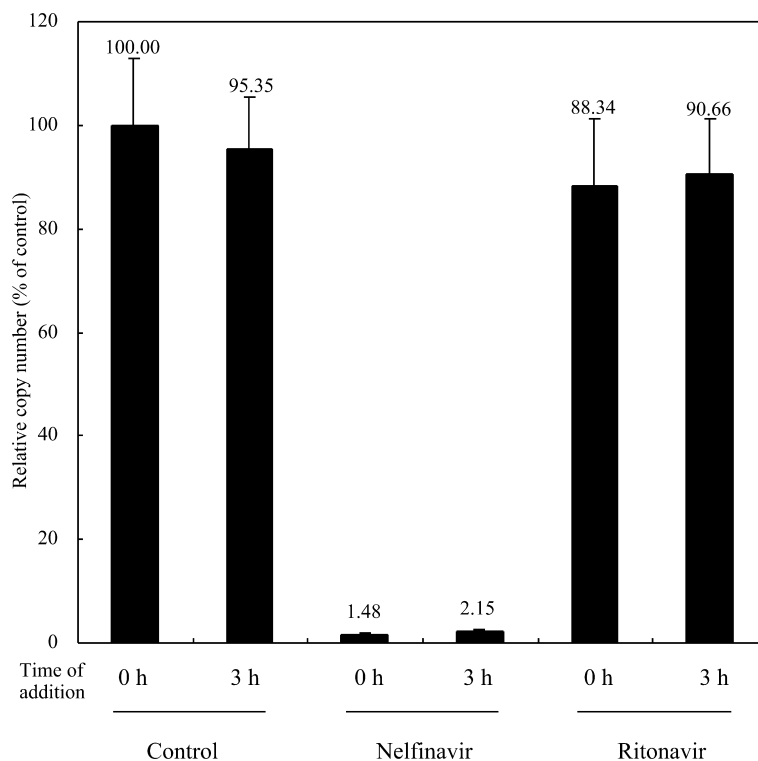


Fig. 4. Real-time RT-PCR for SARS-CoV RNA with various timings of drug addition. Nelfinavir or ritonavir was added at the time of infection or 3 h after infection at the concentration of 10 μ M. Instead of these drugs PBS was added as a negative control. Viral RNA in the culture supernatant was collected 24 h after infection and quantified by the use of a fluorogenic probe. All samples were analyzed in triplicate.

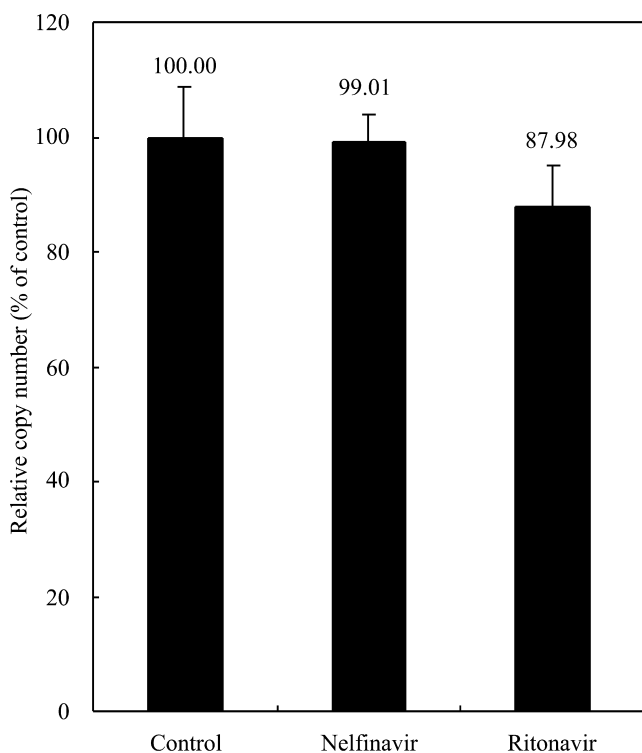


Fig. 5. Entry inhibition assay. To quantify SARS-CoV RNA which entered the cells, Vero E6 cells were pretreated with the drugs and infected with SARS-CoV. Cells were washed with PBS 3 times 3 h after infection. Subsequently viral RNA and 18S ribosomal RNA in the cells were quantified. All samples were analyzed in triplicate.

the molecules expressed after infection with its important role in viral replication [18–20], and the effect of nelfinavir on the main proteinase activity should be investigated. We have cloned, expressed, and purified SARS-CoV main proteinase in order to examine the effect of nelfinavir on this enzyme. Our preliminary study indicated that the activity of the main proteinase was blocked only partially (data not shown), which implies that nelfinavir may interact with some molecule(s) other than the main proteinase to fully inhibit SARS-CoV replication.

Nelfinavir is a very safe and widely used inhibitor of the HIV-1 protease, with strong *in vivo* activity in HIV-infected patients. Nelfinavir is generally used in combination with other antiretroviral medications as part of a highly active antiretroviral regimen (HAART) [21]. When used in this manner, 50–75% of patients who are naive to antiretroviral therapy have plasma HIV RNA levels below the limit of detection in association with an approximate increase of 200 mm^{-3} CD4(+) lymphocytes at 12 months of therapy [22–25]. The most common side effect of nelfinavir is mild diarrhea, which is observed in 15–20% of patients [26]. Nelfinavir is well tolerated by patients with HIV infection. Due to these characteristics, nelfinavir has become one of the most frequently prescribed first line protease inhibitors in the treatment of HIV-infected individuals.

Our studies have clearly shown that nelfinavir can strongly inhibit the replication of SARS-CoV in Vero E6 cells. The safety of this drug for humans has already been established, which constitutes the advantages of nelfinavir even for the clinical use to SARS patients. Our results suggest that nelfinavir should be examined clinically for the treatment of SARS. Moreover, nelfinavir might be a promising lead compound for anti-SARS drugs.

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